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13. ABSTRACT (Maximum 200 Words) Asthma is the most common reason for hospitalization of children in both military and civilian hospitals. In children with asthma, pulmonary exposure to allergen results in damage to bronchioles by invasion of eosinophils. Eosinophils are inflammatory cells, have limited life spans, and must be continually renewed from hematopoietic tissue. We adapted an animal model of asthma to our laboratory for studies of the effect of pulmonary allergen exposure on eosinophil progenitor cells (CFU-eo). These studies have revealed that CFU-eo numbers are elevated in the bone marrow of asthmatic mice following putlmonary allergen exposure. IL-5 is the primary cytokine that regulates eosinophil production and was originally thought to be synthesized exclusively by T lymphocytes. We demonstrated that fibroblastic bone marrow stromal cells produce IL-5 and that stromal cells regulate eosinophil production in vitro. Our working hypothesis is that eosinophil production in asthma is regulated by both bone marrow stromal cells and T lymphocytes. The primary objective of this proposal is to determine the relative role of stromal cells and T lymphocytes. The primary objective of this proposal is to determine the relative role of stromal cells and T lymphocytes in normal and asthmatic eosinophil production. In addition, the effect of inflammatory mediators on stromal cell support of eosinophilopoiesis and the durability of these responses will also be investigated.				
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## Introduction

Asthma is the most common reason for hospitalization of children in both military and civilian hospitals<sup>1-4</sup>. In patients with asthma, pulmonary exposure to allergen results in bronchial hyperresponsiveness and airway inflammation mediated by eosinophils. Eosinophils are inflammatory cells, have limited life spans, and must be continually renewed from hematopoietic tissue. Exposure to allergen has also been correlated with systemic changes in hematopoietic function<sup>5-9</sup>. Our laboratory has demonstrated that initial exposures to allergen are associated with expansion of eosinophil progenitor cells, bone marrow eosinophilia, and progressive accumulation of large numbers of eosinophils in both circulation and lung<sup>10</sup>. These bone marrow changes are regulated by a multi-step process. Whereas, increased bone marrow output of eosinophils is regulated by T lymphocytes, expansion of eosinophil progenitor cells, or CFU-eo, in athymic mice exposed to allergen demonstrates critical importance of additional regulatory mechanisms in the bone marrow. Bone marrow stromal cells appear to be key regulators of CFU-eo expansion. Preliminary data generated during project years 01 and 02 suggest that regulation of eosinophil production by stromal cells is complex and may include negative regulatory signals expressed during steady state eosinophilopoiesis. Excess production of eosinophil production in asthma appears to be due to disruption of this normal homeostatic mechanisms that regulate eosinophil output. These studies take on increased importance because little is actually known about normal regulation of hematopoiesis or the possibility that systemic inflammatory responses may alter these mechanisms.

## Body

### *Original Aims.*

***Research Objective 1: To determine cellular mechanisms that regulate bone marrow eosinophilia following allergen challenge.*** In our initial attempt to dissect regulation of eosinophil development in the bone marrow, we found that bone marrow stromal cells produce IL-5 and supported eosinophil differentiation *in vitro*. IL-5 production by bone marrow stromal cells was upregulated by exposure to IL-1 $\beta$  and this correlated with increased eosinophil differentiation *in vitro*. However, other investigators have documented IL-5 production by CD3+ T lymphocytes in the bone marrow. Experiments in this specific aim will utilize T cell deficient nude mice to determine the role of bone marrow stromal cells and T lymphocytes in eosinophil progenitor cell expansion and differentiation that lead to bone marrow eosinophilia.

***Research Objective 2. To determine the effect of inflammatory mediators associated with asthma on stromal cell function.*** Previous experiments from this laboratory revealed that exposure of stromal cells to IL-1 and IL-4 resulted in failure of their ability to support early events in B lymphocyte development. In this specific aim we will determine the effect of inflammatory mediators that are systemically elevated in asthma on stromal cell cytokine production and function. Specifically, we will investigate stromal cell support of myeloid and lymphoid progenitor expansion.

***Research Objective 3: To determine the kinetics of altered bone marrow cell function in asthma.*** The duration of altered hematopoietic cell production following pulmonary allergen exposure is not known. This question is pertinent to the sensitization and subsequent development of childhood asthma. Establishing the kinetics of this response will be particularly important in understanding whether the bone marrow response changes with repeated exposure to allergen. Experiments in this

specific aim are designed to determine the durability of altered hematopoiesis following single or repeated pulmonary exposure to allergen.

### ***Statement Of Work (Revised 12/31/01)***

**Project Year 01:** In the first year of this project, we will initiate the *in vitro* and *in vivo* studies described in Research Objective 1. Although our laboratory is experienced in rodent surgery and we have an attending veterinarian consulting on this aspect of the project, it is expected that development and conduct of the diffusion chamber experiments will require a total of 30 months and will extend through the second year of the project and be concluded in Project Year 03. Completed studies will be presented at appropriate scientific meetings and prepared for publication in refereed journals.

**Project Year 02:** *In vitro* studies initiated in Project Year 01 (*Research Objective 1*) will continue throughout Project Year 02. We will initiate studies proposed in Research Objective 3 that focus on the durability of effects of repeated *in vivo* allergen dosing regimens on bone marrow function. Completed studies will be presented at appropriate scientific meetings and prepared for publication in refereed journals.

**Project Year 03:** During Project Year 03, we will complete remaining *in vivo* diffusion chamber studies described in Research Objective 1. We will complete studies of long-term allergen exposure and evaluate bone marrow transplantation studies proposed in Research Objective 3. We will repeat studies in each Research Objective 1 and Research Objective 3 as necessary to complete and appropriately document this project in published literature. Completed studies will be presented at appropriate scientific meetings and publications prepared for refereed journals.

### ***Progress Report***

In our statement of work, we proposed initiating studies that were to determine the cellular mechanisms, which regulate bone marrow eosinophilia following allergen challenge (Research Objective 1). These studies focused on the relative roles of bone marrow stromal cells and bone marrow T cells in regulating progenitor cell expansion and expression of eosinophilia following allergen exposure and encompassed both *in vivo* and *in vitro* approaches. In addition, we proposed to evaluate the effects of long-term allergen exposure on bone marrow eosinophilopoiesis (Research Objective 3).

A main focus of our research endeavor during year 02 has been to determine the role of stromal cells in regulation of CFU-eo and eosinophil expansion following allergen exposure. In Research Objective 1, one method proposed to evaluate this important hematopoietic function of stromal cells was to utilize diffusion chamber technology. Initial studies performed in year 01 centered on determining the best method of construction of diffusion chambers to maintain CFU-eo viability. In addition, we determined that enzymatic dissociation of the formed bone marrow clot by trypsin maintained best viability of CFU-eo.

During project year 02 we have continued to focus our efforts on enhancing diffusion chamber construction to maximize CFU-eo numbers. We have determined that a silicone sealant to the rings was determined to maintain the best viability for CFU-eo numbers rather than Millipore cement as originally proposed. In addition, ability of sealant to retain cells in disk without spill over was

evaluated. Cultures were established in 6 well plates under standard conditions. A murine T cell line was cultured alone for 24 hours, and compared to cultures in both non-sealed and sealed diffusion chambers (Figure 1). These data indicate that cells remaining in sealed diffusion chambers are equivalent to cells cultured *in vitro* in the absence of diffusion chambers. Loss of cells is significant in chambers which were plugged but not sealed with silicone.

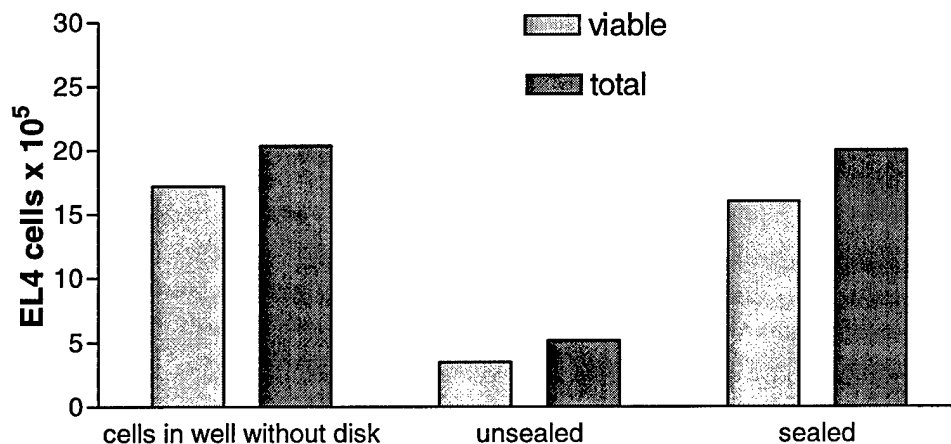


Figure 1

As most bone marrow cells require an optimum concentration *in vitro* to maintain both function and viability, studies were undertaken to determine the best cell concentration to inject into each ring to maintain optimal CFU-eo viability. We performed *in vitro* assays with  $2 \times 10^6$ ,  $4 \times 10^6$ ,  $6 \times 10^6$ ,  $8 \times 10^6$  and  $10^7$  cells injected per ring, cultured in standard tissue culture media for 24 hours then cell number and survival enumerated. In Figure 2, we determined that  $8 \times 10^6$  cells provided maximal return of bone marrow cell number after 24 hours of culture. Viability after 24 hours of culture was determined to be excellent at greater than 80% at all cell concentrations (Figure 3).

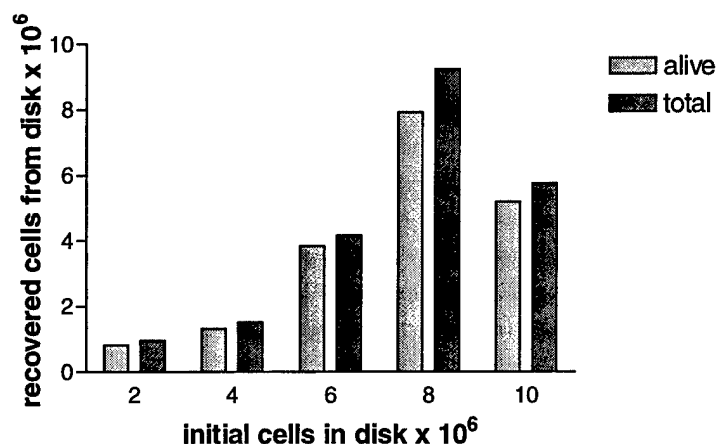


Figure 2

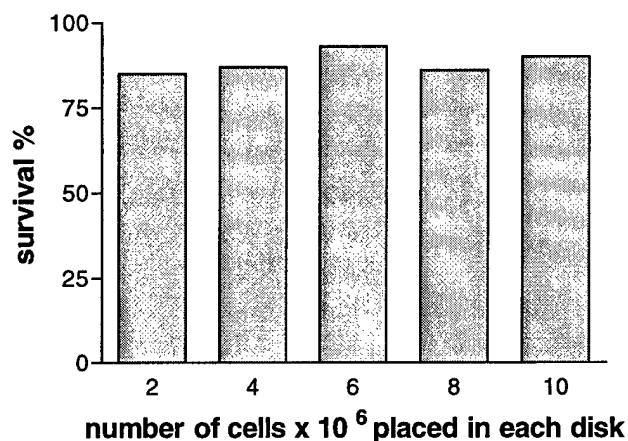


Figure 3

We have also begun inserting diffusion chambers into nude mice to evaluate surgical methods as proposed in research objective 1. To date we have encountered no difficulty with operative implantation of diffusion chambers into nude mice peritoneum. In addition, we have encountered no difficulties with post-operative inflammation or infection. At 24 hours the presence of external clot was noted to be minimal in our model.

We have performed diffusion chamber implantation in both sensitized and unsensitized nude mice with nude mouse bone marrow. Chambers were removed and then established in standard CFU-eo cultures. In both unsensitized and sensitized nude mice we unexpectedly obtained results, which indicated that no CFU-eo were growing in culture. These results mimic *in vitro* results obtained in which stromal cells are co-cultured with bone marrow cells in CFU-eo conditions. (Data not shown.) In these *in vitro* studies we have determined that stromal cells unexpectedly inhibited CFU-eo formation completely. However, before concluding that our *in vivo* diffusion chamber method has also demonstrated stromal cell inhibition of CFU-eo colony formation, we will undertake studies evaluating whether it is feasible to avoid bone marrow cell stress induced by trypsinization of chambers and proceed directly to CFU-eo cultures after chamber removal from nude mice.

Studies performed during project year one demonstrated that stromal cells functionally inhibited CFU-eo colony formation. These studies focused upon the inflammatory cytokine IL-1. IL-1 stimulated stromal cells appeared to increase production of a CFU-eo suppressive factor. Antibody inhibition studies determined that this stromal cell induced suppressive factor was IL-4. In addition, direct exposure of CFU-eo cultures to IL-4 resulted in a dose dependent suppression of CFU-eo colony formation. In addition, it was determined that T cells were not required for observed suppression of CFU-eo colony formation. During project year 2, these studies have been replicated. These studies were selected for presentation at the American Academy of Allergy, Asthma and Immunology Annual Meeting, March 2004.

In light of these findings, studies were undertaken during project year 2, to determine if stromal cells were capable of producing IL-4. In the first study, stromal cells were unstimulated or stimulated with 10 ng/ml IL-1 and intracellular staining with PE conjugated anti-murine IL-4 antibody. In **figure 4**, isotype control antibody staining is indicated in gray, unstimulated stromal

cells stained with anti-IL-4 antibody is outlined in red and IL-1 stimulated, anti-IL-4 antibody stained stromal cells are indicated in blue. These data suggest that stromal cells are capable of IL-4 protein production and that this production is increased after stromal cell stimulation with inflammatory cytokine IL-1.

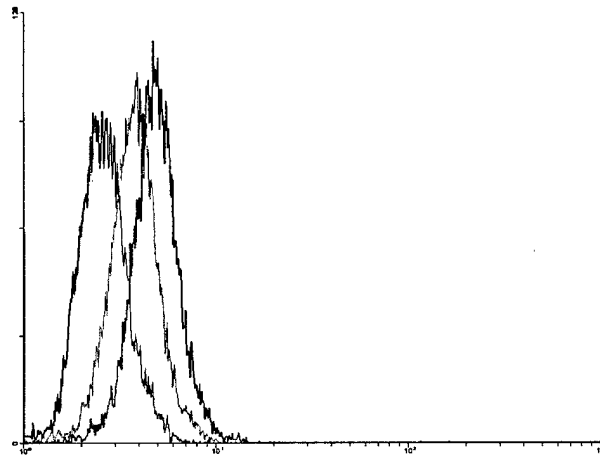


Figure 4

As the finding that stromal cells are capable of producing IL-4 protein is a novel finding, confirmatory studies were also performed during project year 2. In these studies, stromal cells were unstimulated or stimulated with IL-1 and conditioned media collected and concentrated. Detection of IL-4 in 10X concentrated conditioned media was performed utilizing an IL-4 specific ELISA plate. In this study, no IL-4 protein was detected in stromal cell culture supernatant. This finding, however, was not unexpected, as we had previously demonstrated that IL-5 protein production by stromal cells occurs at low levels and is not directly detectable in stromal cell supernatants *in vitro*<sup>11</sup>. Other investigators have demonstrated that stromal cell cytokine production usually occurs at low levels as secretion is highly localized in the bone marrow to hematopoietic cells which are adherent to stromal cells<sup>12</sup>. To enhance recovery of IL-4 protein from stromal cells, we utilized GolgiStop 18 hours prior to utilizing stromal cells to prevent protein excretion. S10 and S17 stromal cells were then lysed and centrifuged. Lysate supernatants were then applied to an IL-4 specific ELISA plate. In **Figure 5**, IL-4 protein production is demonstrated at low levels in unstimulated stromal cells. After IL-1 stimulation of stromal cells there is a significant increase in IL-4 protein production.

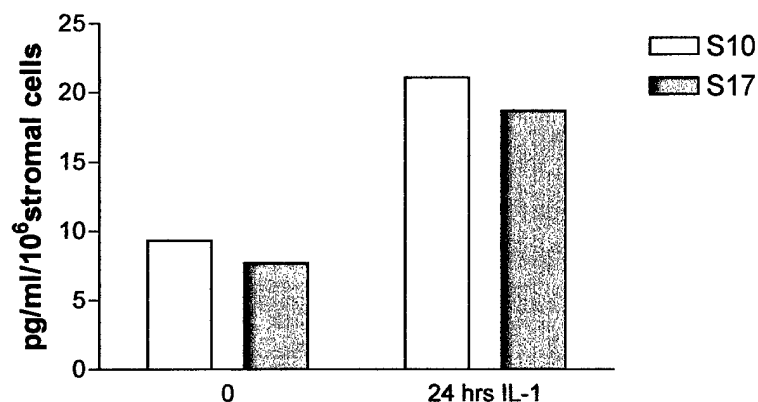


Figure 5.

We hypothesized that inflammatory mediators released from the lung during asthma sensitization affect bone marrow hematopoietic function. We have previously demonstrated that IL-1 a systemically released inflammatory cytokine during asthma increases stromal cell production of IL-5.<sup>11</sup> This increase in stromal cell production of IL-5 is capable of supporting increased eosinophilopoiesis *in vitro*<sup>11</sup>. Substance P is another inflammatory mediator released from sensory neurons following allergen exposure and contributes to pathologic features of asthma, including plasma leakage, mucous secretion and eosinophil chemotaxis to the lung<sup>13-18</sup>. The neurokinin, substance P, is also known to broadly alter hematopoietic function, but has not previously been considered in the context of hematopoietic responses in asthma<sup>19-22</sup>. This mediator is of particular interest to our laboratory because it has been proposed to alter both bone marrow stromal cell function and T cell function and is released from sensory neurons present in the bone marrow<sup>20-27</sup>.

In this preliminary study of the effect of substance P on CFU-eo colony formation, 50 µg/ml substance P was added to CFU-eo cultures. The addition of substance P to CFU-eo cultures resulted in 21% reduction in the number of CFU-eo formed following a single exposure at the initiation of cultures (Figure 6).

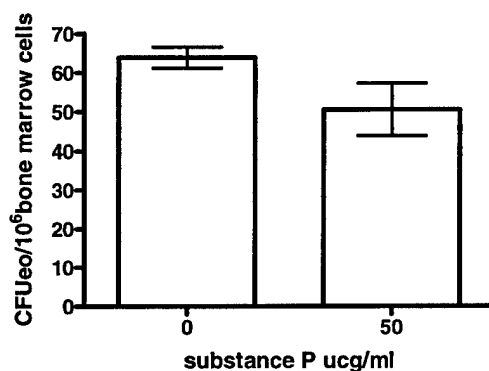


Figure 6

Substance P is known to be labile and is undetectable in cultures after a few hours. For that reason, we exposed CFU-eo to 37.5 µg/ml SP at 0, 24 and 48 hours after initiation of culture and colonies were scored on day 7. As shown in figure 7, repeated exposure to SP during the first 48 hours of culture resulted in complete abrogation of colony formation.

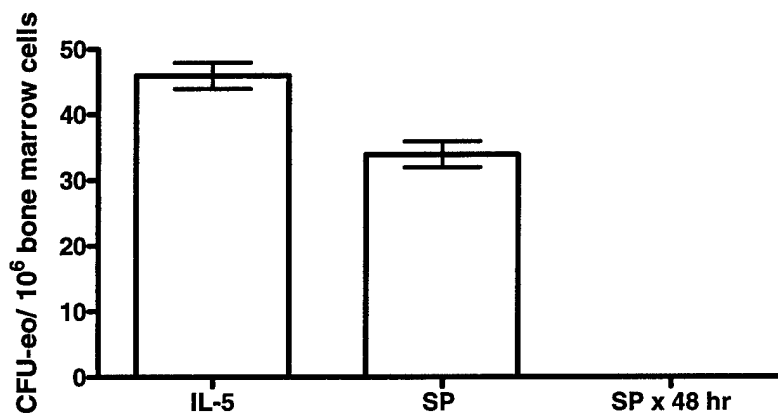
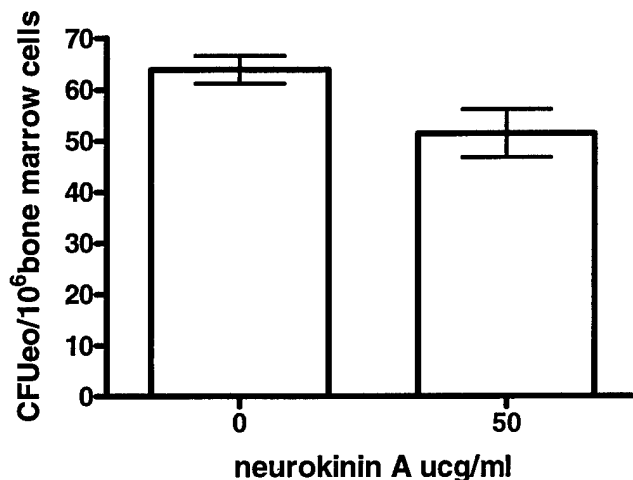


Figure 7.

Neuronkinin A another neurokinin has described effects on hematopoiesis<sup>28</sup>. Elevated levels of neurokinin A have also been demonstrated in asthmatic individuals. In this preliminary study, we evaluated the direct effect of a single exposure of neurokinin A on eosinophil progenitor cells and found that neurokinin A (50 µg/ml, Sigma) also inhibited CFU-eo formation in vitro (**Figure 8**; 19.4% reduction).



**Figure 8.**

We have also initiated *in vivo* experiments to determine the effect of substance P on eosinophilopoiesis during asthma. In this preliminary experiment, athymic mice were exposed to saline or ovalbumin. Substance P receptor antagonist, CP-99994 was given by IP injection to mice on days 9-12 of the sensitization period. Bone marrow was removed on day 13 of the sensitization period and CFU-eo cultures were established. Preliminary data demonstrate that mice given the substance P antagonist had increased numbers of CFU-eo as compared to unexposed nude mice (data not shown). These data suggest that substance P also has an inhibitory effect on CFU-eo colony formation *in vivo*.

Our initial studies were to determine the kinetics of eosinophil progenitor cell expansion in the bone marrow of athymic mice following exposure to allergen. Experiments performed demonstrated that athymic nude mice had significant CFU-eo expansion, which peaks four days following initial intranasal sensitization with ovalbumin and returns to baseline by day 18. We have attempted to determine which cytokine signals are responsible for the accelerated eosinophilopoiesis noted during asthma. In project year one, we have determined that IL-5 is not responsible for increased CFU-eo production in the bone marrow during asthma sensitization. This work has now been published in the *Journal of Immunology* and is included in the appendix<sup>10</sup>. We have also investigated whether GM-CSF, a cytokine known to support eosinophilopoiesis, is responsible for increased CFU-eo production noted in our model. Nude mice were sensitized to ovalbumin and some mice were exposed to anti-murine GM-CSF starting on day 9 of the sensitization period. In **figure 9**, no blocking effect of anti-GM-CSF is noted on CFU-eo production. This data demonstrates that GM-CSF is unlikely to be involved in the accelerated production of CFU-eo during asthma.

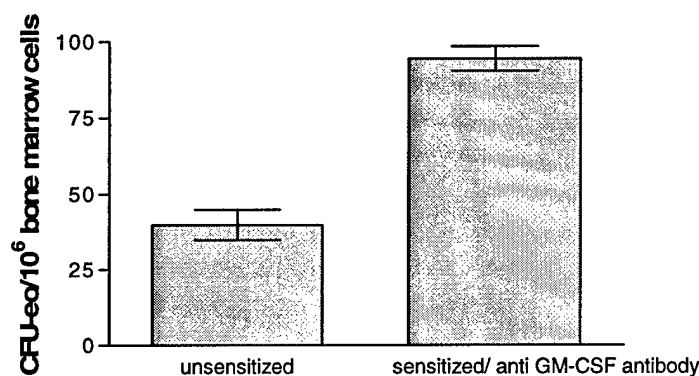


Figure 9.

We have also hypothesized that accelerated CFU-eo production may result from cytokine stimulation of accelerated stem cell production in the bone marrow. Stem cell factor (SCF) is stimulatory of bone marrow stem cell proliferation. Therefore, we chose to investigate the effects of SCF on early eosinophil progenitor production. In this *in vivo* study, athymic nude mice were sensitized to ovalbumin. Anti-murine SCF antibody or its isotype control antibody was administered to mice on days 9-12 of the sensitization period. In **figure 10**, the expected increase in CFU-eo production noted in our model on days 13-14 was not observed in those mice receiving SCF blocking antibody. In fact, minimal CFU-eo production was noted in these mice. These *in vivo* data suggest that SCF may be responsible for increases in CFU-eo production noted during asthma sensitization.

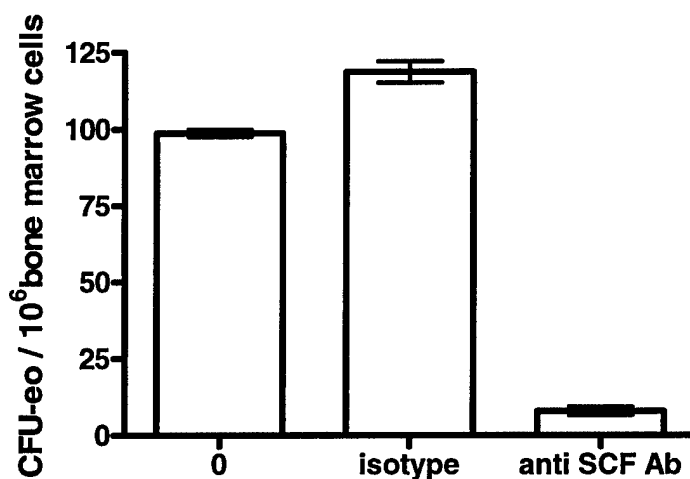
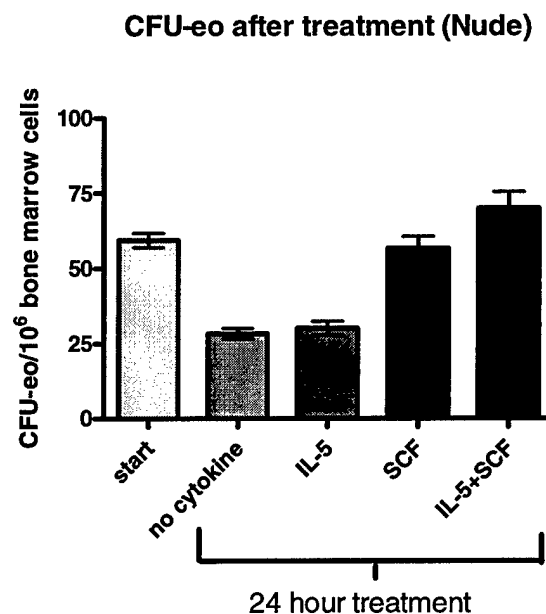
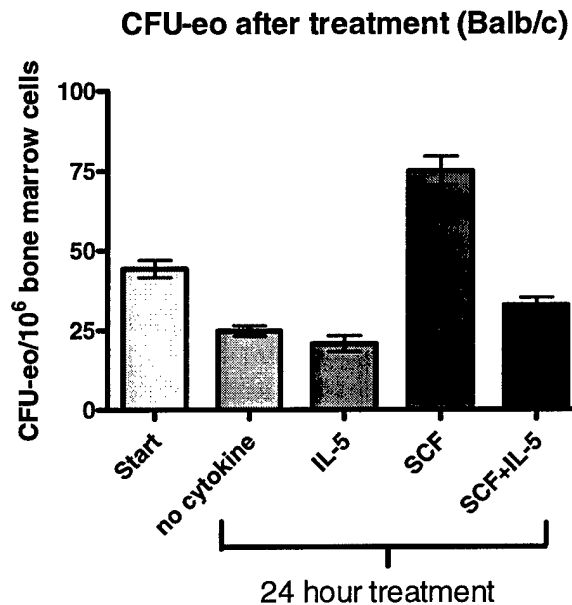


Figure 10

We have also evaluated whether SCF is capable of increasing eosinophil formation *in vitro* (**Figure 11**). Bone marrow cells were removed and cultured in the presence of either IL-5, SCF, or a combination of IL-5 and SCF. Our preliminary data (**Figure 11**) demonstrate that SCF addition resulted in expansion of CFU-eo in *in vitro* 24-hour cultures, however, IL-5 did not stimulate CFU-eo expansion. Taken together with previous results, these data strongly suggest that CFU-eo

expansion observed following allergen exposure is not due to elevated levels of IL-5 as has been previously reported. Interestingly, in uethymic BALB/c mice, addition of IL-5 to SCF containing cultures abrogated CFU-eo expansion, however, this result was not observed in athymic nude mice. Reasons for this difference in response are currently under investigation in our laboratory.



**Figure 11**

We have also proposed to determine the role of T cells in the accelerated eosinophilopoiesis noted during asthma. In project year one, we determined that T cells are critical to the development of the accelerated mature eosinophilopoiesis noted during asthma. These data were published in the *Journal of Immunology* and are included in the appendix<sup>10</sup>. We have undertaken preliminary studies in project year two to identify the mechanism in which T cells perform this critical function in supporting eosinophilopoiesis during asthma. Work is currently underway to establish an *in vivo* and T cell transplant model to facilitate this work.

Currently, we have successfully transplanted wild type balb/c splenic T cells into athymic balb/c nude mice. Balb/c donor mice were exposed to saline (unsensitized) or sensitized to ovalbumin. In these preliminary studies, splenic T cells were then removed from mice on day 11 of the sensitization period and purified by immunomagnetic bead separation.  $10^7$  T cells were injected IP into unsensitized athymic nude balb/c mice. Bone marrow was removed 4 days after transplantation and CFU-eo cultures established (Figure 12). These preliminary data suggest that T cells may be participating in suppression of CFU-eo at steady state conditions of hematopoiesis and early in the sensitization period.

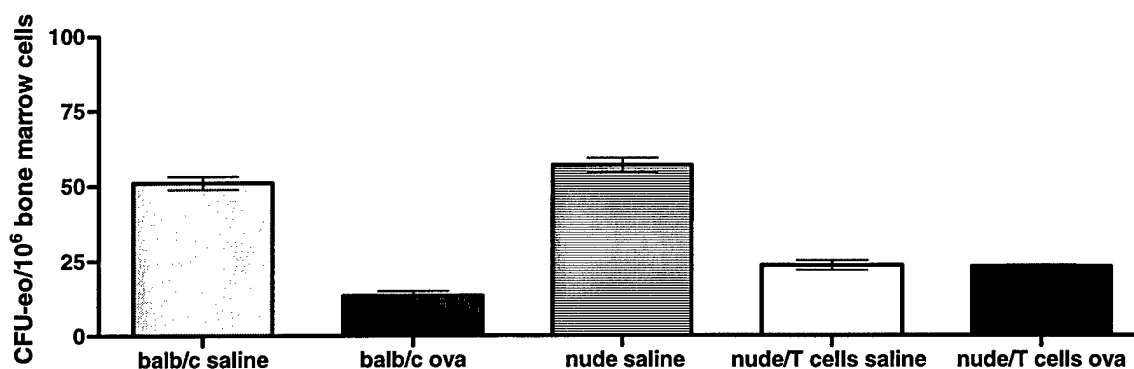


Figure 12.

*In vitro* studies investigating the role of T cells in supporting bone marrow eosinophilopoiesis are underway. In this preliminary study, splenic T cells were obtained from both ovalbumin exposed and saline exposed wild type balb/c mice on day 14 of the sensitization period, and purified by immunomagnetic bead separation. Athymic nude mouse bone marrow was placed into CFU-eo cultures with and without unexposed and exposed wild type balb/c T cells (Figure 13). T cells added to cultures represent 10% of the total cell population in culture. These preliminary *in vitro* data once again demonstrate that CFU-eo cultures established with T cell reconstituted athymic bone marrow have fewer CFU-eo numbers.

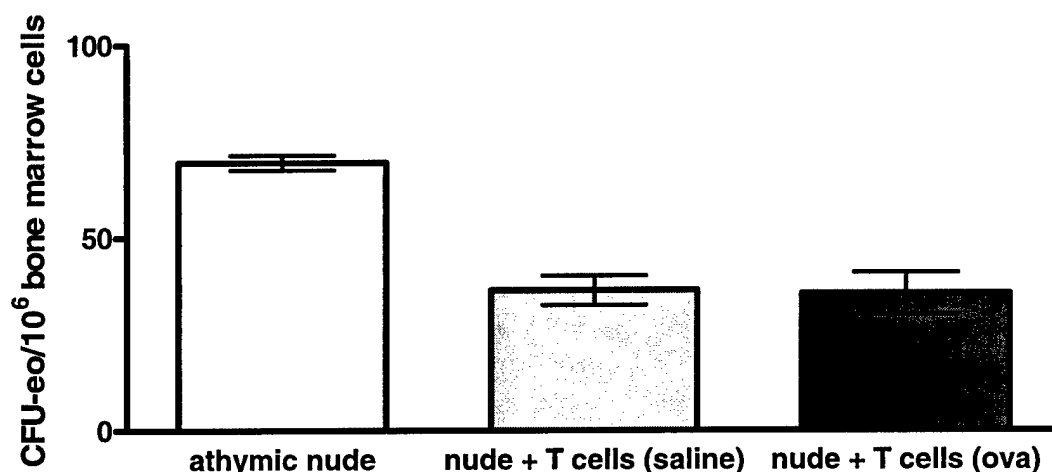


Figure 13

Previous data demonstrated that T cells do not participated in the accelerated CFU-eo production seen during sensitization. In addition, our published data demonstrates that T cells accelerate mature eosinophil production. Studies are ongoing to confirm these findings and to determine if numbers of T cells transplanted effect these findings. In addition, we are seeking to confirm that athymic nude bone marrow is reconstituted with T cells after transplantation. Other studies are currently aimed at determining the effect of T cell transplantation on athymic nude mouse mature eosinophil production. More interestingly, we will pursue studies in project year 3 aimed at determining if IL-4 production by T cells is responsible for suppression of CFU-eo numbers during early asthma.

In research object 3, we proposed to determine the durability of the bone marrow response in asthma by establishing a repetitive challenge model more close mimicking chronic repetitive exposure to allergen in childhood asthma. We have initiated repetitive challenge studies as proposed in balb/c mice. Exposure period was weekly over 2 months. In these studies, no evidence of chronic altered production of eosinophil progenitors or mature eosinophils was demonstrated. However, a very recent publication by Shinagawa and Kojima<sup>28</sup> suggest that murine strain difference may be responsible for these findings. A/J mice were demonstrated in this study to have asthma features consistent with chronic asthma in humans. These findings included airway wall thickening, and persistent airway hyperreactivity<sup>28</sup>. The contribution of bone marrow eosinophil production to the development of these chronic asthma changes was not determined. We have recently initiated studies utilizing Shinagawa's published methods in A/J mice, which are focused on answering whether bone marrow eosinophilopoiesis is altered during chronic asthma.

### Key Research Accomplishments: Year 1

- Eosinophil progenitor cell expansion is regulated by a T-cell independent mechanism.
- Eosinophil progenitor expansion is not regulated by IL-5.
- Bone marrow T cell numbers remain stable during allergen sensitization.
- IL-5 producing cell numbers in the bone marrow are unchanged after sensitization.
- Surgical implantation of diffusion chambers is achieved without significant accompanying inflammation.

- Determined that diffusion chamber apparatus does not affect CFU-eo numbers in culture.
- Determined that removal of cells from diffusion chamber will be accomplished by trypsinization.
- Stromal cells inhibit CFU-eo formation.
- Stromal cell conditioned media inhibits CFU-eo formation in a dose dependent fashion.
- Exposure of stromal cells to inflammatory cytokines IL-1 and IL-4 intensifies CFU-eo inhibition.
- IL-4 and TGF- $\beta$  directly inhibit CFU-eo progenitor cell formation.
- There is no direct effect of IL-1 or IL-6 on CFU-eo progenitor cells.
- Stromal cell conditioned media inhibition of CFU-eo is reversed by anti-IL-4 antibody
- Bone marrow T cells are not indirectly involved in CFU-eo inhibition by stromal cells conditioned medium.

### Key Research Accomplishments: Year 2

- Determined best method of diffusion chamber construction to enhance CFU-eo viability.
- Determined optimal cell number to inject into diffusion chambers to enhance CFU-eo recovery and viability.
- Determined utilizing diffusion chamber technology that stromal cells *in vivo* suppress CFU-eo colony formation.
- Confirmed suppressive effect of stromal cells on CFU-eo formation is in part due to IL-4.
- Confirmed suppressive effect of IL-4 on CFU-eo does not require T cells.
- Determined that stromal cells are capable of producing IL-4 protein.
- Determined that stromal cells are capable of increasing IL-4 protein production after stimulation with inflammatory cytokines present in asthma, such as IL-1.
- Determined that substance P directly inhibits CFU-eo formation *in vitro*.
- Determined that neurokinin A directly inhibits CFU-eo formation *in vitro*.
- Determined that administration of substance P receptor antagonist *in vivo*, results in increased CFU-eo numbers, which reflects the role of substance P as a potential inhibitor of CFU-eo colony formation.
- Determined that GM-CSF does not contribute to accelerated CFU-eo production during asthma sensitization.
- Determined that SCF is partially responsible for accelerated CFU-eo production noted *in vivo* during asthma sensitization.
- Determined that SCF is synergistic with IL-5 in accelerating mature eosinophil production.
- Determined that reconstitution of athymic nude mice with wild type T cells results in suppressed CFU-eo numbers *in vivo*.
- Determined that reconstitution of athymic nude mouse bone marrow with wild type T cells suppresses CFU-eo numbers *in vitro*.
- Determined that balb/c mice do not have functional alteration in bone marrow eosinophilopoiesis in a long-term allergen challenge model.

**Reportable Outcomes****Publications resulting from this award.**

Hogan MB, Weissman DN, Hubbs AF, Landreth KS. Regulation of eosinophilopoiesis in a murine model of asthma. J Immunol 2003 171:2644-51.

**Abstracts presented.**

Hogan MB, Weissman DN, Zhuang ZZ, Landreth KS Bone marrow CFU-eosinophil (CFU-eo) production in a murine asthma model. American Academy of Allergy Asthma and Immunology Annual Meeting New York, NY, March 2002

Hogan MB, Weissman DN, Gibson LF, Piktel D, Welch J, Landreth KS. Role of bone marrow T cells in eosinophil production of asthma. AAAAI National meeting, Denver, CO. March 2003.

KS Landreth, D Piktel, LF Gibson, DN Weissman, J Welch, MB Hogan. Regulation of eosinophilopoiesis by stromal cells is modulated by inflammatory cytokines. International Society of Experimental Hematology Annual Meeting. Paris, France, July 5-8 2003

Hogan MB, Landreth KS. Bone Marrow Function in Development of Childhood Asthma. Peer Reviewed Medical Research Program (PRMRP) 2003 Programmatic Review Meeting, August, 2003.

Hogan MB, Piktel D, Simpson R, Gibson LF, Welch JE, Landreth KS. Suppression of CFU-eo Formation by Bone Marrow Stromal Cells and IL-4. AAAAI National Meeting, San Francisco, CA, March 2004.

Landreth KS, Hogan MB, Gibson LF, Weissman DN, Piktel D. Altered bone marrow function associated with development of asthma. Department of Defense Peer Reviewed Medical Research Program Investigators Meeting. Puerto Rico, USVI (submitted).

**Invited Presentations.**

The role of the bone marrow in the onset of asthma. Department of Pediatrics Grand Rounds. WVU, May 21, 2003.

The role of the bone marrow in the onset of asthma. Pediatric Allergy/Immunology Basic Science Seminar. Northwestern University, Chicago, IL. August 21, 2003

The role of the bone marrow in the onset of asthma. Department of Pediatrics Grand Rounds. Children's Memorial Hospital, Chicago, IL August 22, 2003.

The role of the bone marrow in the onset of asthma. Allergy/Immunology Seminar. Rush Presbyterian Hospital, University of Chicago, Chicago, IL August 22, 2003.

The role of the bone marrow in the onset of asthma. West Virginia Chapter, American Lung Association Annual Meeting. Morgantown, WV, October 2, 2003

### **Pending Future Funding.**

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Department of Defense. Peer Reviewed Medical Research Program. PR043167. Regulation of bone marrow response in asthma. **Co-Principle Investigator** (Hogan, Mary Beth), (Principle Investigator, Kenneth S. Landreth). Direct Costs. \$1,368,229. Submitted 3/16/04

### **Personnel Receiving Pay from the Research Effort**

Mary Beth Hogan, MD  
Kenneth S. Landreth, Ph.D.  
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Deborah Piktel, B.A.

### **Conclusions:**

Asthma is a complex disease in which multiple mediators and cell types contribute to the pathogenesis of airway compromise. It has been recently appreciated that asthma also has systemic effects upon bone marrow regulation of hematopoiesis, in particular eosinophilopoiesis. The bone marrow environment consists of hematopoietic cells, stromal cells, mature end cells and T lymphocytes. Inflammatory mediators generated and released from pulmonary tissue, and potentially produced locally in the bone marrow during the development of asthma have the potential to exert regulatory control on bone marrow cells. In our own studies, numbers of eosinophil progenitor cells (CFU-eo) were found to be initially depleted in the bone marrow, followed by a transient rebound to supra-normal levels. This rise in numbers of CFU-eo following allergen stimulation appeared to be regulated by stromal cells.

In the experiments presented here, we demonstrate that stromal cells from untreated mice actually secrete cytokines that inhibit eosinophil production. This inhibitory function is accelerated by exposure to IL-1, an inflammatory cytokine released systemically following allergen exposure *in vivo*. In addition, data presented in this report suggest that other inflammatory mediators, such as substance P present in the bone marrow may also suppress eosinophilopoiesis. These data suggest that the decline of bone marrow CFU-eo that follows allergen stimulation may be due to increased suppression of eosinophilopoiesis rather than loss due to increased demand for mature eosinophils as previously reported from our laboratory. Subsequent rebound of CFU-eo and eosinophils, which has been observed following allergen exposure, is likely due to normal feedback mechanisms that regulate eosinophil homeostasis.

Our interest in childhood asthma has led us to investigate events in eosinophilopoiesis during the sensitization phase of asthma. This investigation has led us to propose that eosinophilopoiesis is

regulated in a step-wise manner by bone marrow stromal cells and T lymphocytes. This hypothesis is the basis for the design of experiments to be done in the third year of this grant.

Experiments to be completed in the next year of this grant will be focused on understanding both the downregulation of CFU-eo generation observed in the presence of stromal cells and the significant increases in CFU-eo production during both steady state hematopoiesis and during the initiation of asthma. We will continue studies aimed at identifying CFU-eo downregulatory cytokines produced by stromal cells and potentially T cells. Investigations of alternate cytokine signals, which may accelerate CFU-eo expansion, such as SCF, are also planned. In addition, we will follow up on preliminary studies performed in project year 02 which suggest that substance P may be an important downregulatory signal in eosinophilopoiesis. In Project Year 03 we will continue to evaluate the effect of repeated administration of allergen on eosinophil production in the bone marrow as described in Research Objective #3.

Currently there are no long-term options for intervening in the process of allergen sensitization and development of childhood asthma. Studies proposed in this grant will determine the regulatory mechanisms of bone marrow eosinophil production at both steady state and as altered in the disease state of asthma. Special emphasis on investigating the role of both bone marrow stromal cells and T cells in eosinophilopoiesis is ongoing. Preliminary data suggest that the role of T cells in eosinophilopoiesis may be complex, with T cells providing downregulatory signals early in eosinophil progenitor formation, but providing signals accelerating mature eosinophil production. In addition, ongoing studies suggest that stromal cells may provide key downregulatory signals, such as IL-4, which control eosinophil production under normal and inflammatory conditions. Elucidation of the normal downregulatory mechanisms of eosinophil production may lead to strategies for childhood asthma that ultimately inhibit disease development or progression.

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## APPENDIX

### Manuscript:

Hogan MB, Weissman DN, Hubbs AF, Gibson LF, Piktel D, Landreth KS. Regulation of eosinophilopoiesis in a murine model of asthma. J Immunol 2003 171:2644-51.

# Regulation of Eosinophilopoiesis in a Murine Model of Asthma<sup>1</sup>

Mary Beth Hogan,<sup>2\*</sup> David N. Weissman,<sup>§</sup> Ann F. Hubbs,<sup>§</sup> Laura F. Gibson,<sup>\*†‡</sup> Debra Piktet,<sup>†‡</sup> and Kenneth S. Landreth<sup>\*†‡</sup>

Eosinophilic inflammation plays a key role in tissue damage that characterizes asthma. Eosinophils are produced in bone marrow and recent observations in both mice and humans suggest that allergen exposure results in increased output of eosinophils from hemopoietic tissue in individuals with asthma. However, specific mechanisms that alter eosinophilopoiesis in this disease are poorly understood. The current study used a well-characterized murine animal model of asthma to evaluate alterations of eosinophil and eosinophil progenitor cells (CFU-eo) in mice during initial sensitization to allergen and to determine whether observed changes in either cell population were regulated by T lymphocytes. Following the first intranasal installation of OVA, we observed sequential temporal elevation of eosinophils in bone marrow, blood, and lung. In immunocompetent BALB/c mice, elevation of bone marrow eosinophils was accompanied by transient depletion of CFU-eo in that tissue. CFU-eo rebounded to elevated numbers before returning to normal baseline values following intranasal OVA exposure. In T cell-deficient BALB/c nude (BALB/c<sup>nu/nu</sup>) mice, CFU-eo were markedly elevated following allergen sensitization, in the absence of bone marrow or peripheral blood eosinophilia. These data suggest that eosinophilia of asthma results from alterations in two distinct hemopoietic regulatory mechanisms. Elevation of eosinophil progenitor cells in the bone marrow is T cell independent and likely results from altered bone marrow stromal cell function. Differentiation of eosinophil progenitor cells and phenotypic eosinophilia is T cell dependent and does not occur in athymic nude mice exposed to intranasal allergen. *The Journal of Immunology*, 2003, 171: 2644–2651.

Asthma is characterized by reversible airway hyperactivity and progressive airway inflammation. In patients with asthma, this pulmonary reaction to inhaled allergen has been divided into early phase responses and late phase responses. The early phase response to inhaled allergen results in mast cell degranulation, release of vasoactive and bronchoconstrictive cytokines, restricted airflow, and wheezing (1). Mediators released by mast cells are chemotactic and initiate pulmonary infiltration of lymphocytes, neutrophils, and eosinophils following allergen exposure (2, 3). It is the accumulation of activated eosinophils during the late phase response to allergen exposure that ultimately results in progressive inflammatory tissue damage. In addition, pulmonary eosinophilia in response to allergen challenge is associated with elevated levels of eosinophil-derived cytokines in both the lung and peripheral blood (4, 5).

The eosinophilic inflammatory response is not limited to pulmonary tissue. Increased numbers of eosinophils have also been noted in bone marrow of atopic patients with asthma (6, 7). In a murine model of asthma, transient bone marrow eosinophilia was demonstrated following airway sensitization to OVA and following subsequent allergen challenge (8–10). In both cases, bone mar-

row eosinophilia was followed by peripheral blood and pulmonary eosinophilia (8, 9) and circulating eosinophils appeared to be newly produced cells emigrating from the bone marrow (11).

The aim of the present study was to better define the temporal sequence of events that lead to bone marrow eosinophilia following initial airway exposure to allergen in this animal model and to determine cellular mechanisms that regulate altered eosinophil production in response to allergen exposure. Following the initial sensitizing airway exposure to OVA, we observed sequential eosinophilia in bone marrow, peripheral blood, and lungs of mice. Eosinophil progenitor cells (CFU-eo)<sup>3</sup> in the bone marrow were initially depleted in the bone marrow of mice exposed to allergen, followed by rebound in CFU-eo numbers to greater than baseline values before returning to the level found in untreated controls. To determine the requirement for T lymphocytes in this bone marrow response to initial allergen exposure, T cell-deficient BALB/c nude mice were evaluated using the same exposure regimen. In nude mice, CFU-eo were markedly increased immediately following allergen sensitization, in the absence of detectable eosinophilia in bone marrow or peripheral blood. These findings confirm the importance of T lymphocyte function in bone marrow and pulmonary eosinophilia of asthma, but reveal that altered kinetics of eosinophil progenitor cells in the bone marrow is T cell independent and likely due to altered bone marrow stromal cell function in response to allergen exposure.

## Materials and Methods

### Mice

Four- to 6-wk-old, female, BALB/c<sup>+/+</sup> or athymic BALB/c<sup>nu/nu</sup> (nude) mice were obtained from Taconic Laboratories (Germantown, NY). All mice were housed in autoclaved microisolator cages (Lab Products, Maywood, NJ) and autoclaved food and acidified water (pH 2.8) were provided

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<sup>3</sup> Abbreviations used in this paper: CFU-eo, CFU eosinophil; i.n., intranasal; BAL, bronchoalveolar lavage.

ad libitum. A 12-h light-dark cycle was provided. All procedures were approved by the West Virginia University Animal Care and Use Committee that follows the *Guide for the Care and Use of Laboratory Animals*.

### Allergen sensitization

Pulmonary sensitization to OVA has been previously described in detail (8). Briefly, in each experiment at least four mice were injected i.p. with 100 mg/kg OVA (Sigma-Aldrich, St. Louis, MO) suspended in a saturated solution of aluminum potassium sulfate (alum; Sigma-Aldrich) in sterile distilled water on day 0. For i.p. injections, OVA (0.5 mg/ml) was suspended in 10 ml of endotoxin-free 0.9% saline and equal volumes of working solutions of OVA and alum mixed, adjusted to pH 6.5, and allowed to precipitate for 30 min. The precipitate was centrifuged at 1800 rpm at room temperature, supernatant was removed, and precipitate was resuspended in 10 ml of endotoxin-free saline (8). On day 10, mice were exposed to 25  $\mu$ l of OVA dissolved in endotoxin-free sterile saline delivered into the lung by intranasal (i.n.) deposition under ketamine anesthesia and a second i.p. administration of OVA (0.5 mg/ml) coprecipitated with alum as described above (8). In some experiments (Fig. 3), mice received only i.n. OVA without the usual accompanying i.p. exposure. In all experiments, control mice were handled identically and administered saline i.p. and i.n. on the same schedule.

### Bone marrow and peripheral blood

Mice were euthanized by CO<sub>2</sub> asphyxiation, the peritoneal cavity was opened, and peripheral blood was obtained directly from the inferior vena cava using a heparinized tuberculin syringe. Total white blood cell counts were obtained using a Coulter counter and peripheral blood smears were made to establish a differential white blood cell count. Bone marrow was obtained by flushing femora with  $\alpha$ MEM (Life Technologies, Gaithersburg, MD) supplemented with 1% FCS (Summitt Biotechnology, Fort Collins, CO) using a syringe fitted with a 23-gauge needle. Total white blood cell counts were evaluated microscopically using a hemocytometer. Bone marrow ( $10^5$ ) or peripheral blood cells were cytocentrifuged onto cleaned glass slides and stained with May-Grünwald-Giemsa (Sigma-Aldrich) for enumeration of eosinophils.

### CFU-eo cultures

Eosinophil progenitors were evaluated using standard in vitro CFU assays (CFU-eo). CFU-eo were established with  $7.5 \times 10^5$  bone marrow cells/ml suspended in Methocult M3234 (Stem Cell Technologies, Vancouver, Canada) with or without 10 ng/ml IL-5 (BioSource International, La Jolla, CA). Colonies of >50 cells were counted after 7 days under a stereomicroscope and colony numbers were corrected to absolute values. Colonies were picked, cytocentrifuged, and stained with May-Grünwald-Giemsa to verify the presence of eosinophils.

### Bronchoalveolar lavage (BAL)

Mice were euthanized by CO<sub>2</sub> asphyxiation, the peritoneal cavity was opened, and the trachea was exposed. The trachea was cannulated with a 22-gauge i.v. catheter. PBS (500  $\mu$ l of PBS) was injected and withdrawn from the lung using a tuberculin syringe. This procedure was repeated five times. A white blood cell count of BAL fluid was evaluated microscopically by hemocytometer. Cells were then cytocentrifuged onto clean glass slides and stained with May-Grünwald-Giemsa stain and cell differential counts were obtained. After BAL, lungs were inflated with 1 ml of 10% neutral-buffered Formalin (Sigma-Aldrich). Lungs were embedded in paraffin, sectioned, and stained with H&E and 0.5% chromotrope 2R for identification of eosinophils.

### ELISA

Murine anti-OVA IgE Ab was detected in plasma samples using an IgE-capture ELISA. The following reagents were used, with appropriate washing between incubations: monoclonal anti-mouse IgE (BD Pharmingen, San Diego, CA), PBS/1% skim milk; plasma samples diluted 2-fold over a range from 1/50 to 1/3200, OVA (25  $\mu$ g/ml; Sigma-Aldrich), rabbit anti-OVA-HRP conjugate (Rockland Immunochemicals, Gilbertville, PA); and tetramethylbenzidine substrate solution (Kirkegaard & Perry, Gaithersburg, MD). After incubation for 30 min at room temperature, reactions were stopped (Tetramethylbenzidine Stop Solution; Kirkegaard & Perry) and color development evaluated as OD<sub>450</sub> using an automated plate reader. Specific IgE levels are reported as the reciprocal titer yielding an OD<sub>450</sub> greater than two times background. A positive titer was defined as >1:2.

### ELISPOT

Millipore Multiscreen-IP plates (Millipore, Bedford, MA) were coated with 50  $\mu$ l/well 10  $\mu$ g/ml solution TRFK-5 Ab (Mabtech, Cincinnati, OH) diluted in coating buffer (0.1 M Na<sub>2</sub>CO<sub>3</sub>, 0.1 M NaHCO<sub>3</sub>, pH 9.6). Plates were incubated overnight at 4°C in a moist chamber, washed three times with 200  $\mu$ l of sterile PBS, and blocked by addition of 100  $\mu$ l/well  $\alpha$ MEM containing 10% FCS for 1 h at room temperature. Medium was then removed from wells and  $2 \times 10^5$  bone marrow cells in 100  $\mu$ l of medium added. Cells were incubated overnight at 37°C, culture medium was removed, and wells were washed six times with 0.05% Tween 20 in PBS (PBST; Sigma-Aldrich), one microgram per milliliter TRFK-4 anti-IL-5 Ab (Mabtech) was diluted in 0.5% BSA/0.05% Tween 20 in PBS and 100  $\mu$ l/well was incubated for 2 h at room temperature. Plates were then washed six times with PBST, allowing 15-min incubation at room temperature with each PBST wash. One hundred microliters per well Vectastain Elite (Vector Laboratories, Burlingame, CA) was added to all wells, incubated for 1 h at room temperature, and washed a final time with PBST and three washes with normal PBS. One hundred microliters per well Vector VIP Substrate kit for peroxidase (Vector Laboratories) was added and the plate was developed until spots were visualized. At the termination of development, plates were rinsed for 5 min with deionized water and air dried overnight. Spots were counted using Optimas Imaging Software (MediaCybernetics, Carlsbad, CA).

### In vivo Ab suppression of IL-5 expression

IL-5 production was experimentally blocked in BALB/c<sup>mu/mu</sup> mice by administering 50  $\mu$ g anti-IL-5 mAb TRFK-5 (eBioscience, San Diego, CA) or an isotype-matched control Ig by i.p. injection 1 day before i.n. exposure to allergen (experimental day 9). Intraperitoneal injection of TRFK-5 or the isotype-matched control Ig was repeated daily for 3 days following the initial i.n. allergen exposure. On experimental day 14, mice were euthanized and bone marrow was collected to determine the number of CFU-eo as described above.

### In vitro suppression of CFU-eo formation using TRFK-5 anti-IL-5 Ab

In some experiments, anti-IL-5 Ab (TRFK-5) or an isotype-matched control Ig at the same concentration was added to CFU-eo cultures at 50  $\mu$ g/ml to determine the efficacy of this Ab in neutralizing rIL-5 added to these cultures to stimulate colony formation.

### Statistic analysis

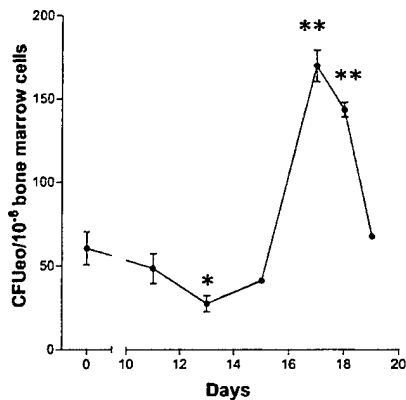
Unless otherwise indicated, all data in this study were analyzed using a one-way ANOVA and Student-Newman-Keuls or Tukey-Kramer comparison testing of ranked means to evaluate the difference among experimental treatment groups. All statistical analysis was performed using GraphPad InStat Software (GraphPad, San Diego, CA).

## Results

### Effect of allergen sensitization on eosinophil populations in the bone marrow

In all experiments, mice received an initial i.p. exposure to OVA (day 0) followed by i.n. exposure to the same allergen on day 10 as described in *Materials and Methods*. This allergen exposure regimen did not result in altered numbers of total nucleated bone marrow cells in any of the experiments presented (data not shown). On the other hand, we consistently noted significant depression of the number of eosinophil progenitor cells, or CFU-eo, 3 days following i.n. installation of allergen (day 13, Fig. 1). This initial depression of bone marrow CFU-eo was accompanied by significant elevation of bone marrow eosinophils (Fig. 2). CFU-eo numbers in bone marrow of allergen-exposed mice rebounded to greater than control values on day 17 (Fig. 1) and returned to baseline values by day 19. Bone marrow eosinophilia in allergen-exposed mice resolved to control values by day 17 (Fig. 2).

In the experimental protocol previously used to establish OVA allergen sensitivity in mice (8), the initial i.n. exposure to OVA on day 10 was accompanied by a second i.p. exposure to the same allergen coprecipitated with aluminum potassium sulfate. To determine whether observed alterations of CFU-eo following allergen sensitization were due to the i.n. deposition of OVA or to the

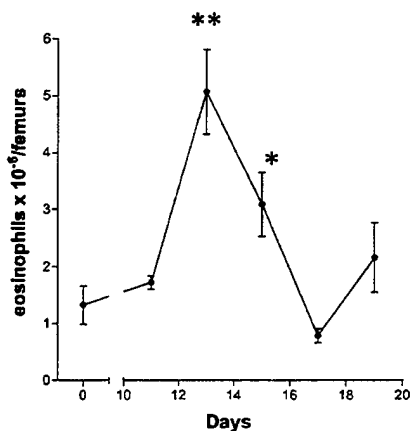


**FIGURE 1.** Kinetics of eosinophil progenitor cells (CFU-eo) during allergen sensitization. Mice were exposed to OVA and alum by i.p. injection on days 0 and 10. Intranasal OVA was delivered on day 10 under light anesthesia. Bone marrow CFU-eo were evaluated as described by incubating  $7.5 \times 10^5$  bone marrow cells in methylcellulose for 7 days in the presence or absence of 10 ng/ml recombinant mouse IL-5. Data presented are the means  $\pm$  SEM of three independent observations. Statistical significance was determined using Student-Newman-Keuls comparison testing of ranked means. Significant differences from control values were recorded on day 13 ( $p < 0.01$ ), day 17 ( $p < 0.001$ ), and day 18. ( $p < 0.001$ ).

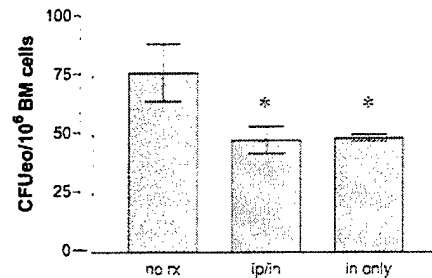
accompanying i.p. exposure, we compared the effect of the traditional exposure regimen to one which utilized i.n. exposure to OVA in the absence of a second i.p. treatment. As shown in Fig. 3, i.n. exposure and i.n. exposure combined with an i.p. exposure to OVA were equally effective in stimulating the observed drop in bone marrow CFU-eo on day 13 of the exposure regimen.

#### *Effect of allergen sensitization on peripheral blood and pulmonary eosinophils*

In mice receiving an initial i.n. exposure to OVA, peripheral blood eosinophilia was not observed until 5 days following i.n. allergen exposure (Fig. 4, day 15) and eosinophilia was not resolved by day 19. Leukocytes were elevated in BAL fluid obtained from these mice on days 11 and 17 as compared with control mice, with



**FIGURE 2.** Kinetics of bone marrow eosinophils during allergen sensitization. Bone marrow cells were cytocentrifuged onto clean glass slides and stained with May-Grünwald-Giemsa. A minimum of 200 bone marrow cells were counted under high-power light microscopy and eosinophil number was determined for each sample. Data presented are the means  $\pm$  SEM of three independent observations. Statistical significance was determined using Student-Newman-Keuls comparison testing of ranked means. Statistical significance was achieved on day 13 ( $p < 0.001$ ) and day 15 ( $p < 0.01$ ).

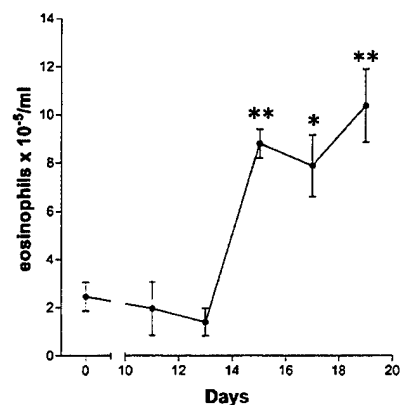


**FIGURE 3.** Effect of route of allergen exposure on bone marrow CFU-eo. Mice were exposed to OVA and alum by i.p. injection on day 0. On day 10, mice received either i.n. OVA alone or i.n. OVA accompanied by a second i.p. treatment with OVA and alum and were compared with untreated controls. Bone marrow CFU-eo were evaluated as described by incubating  $7.5 \times 10^5$  bone marrow cells in methylcellulose for 7 days in the presence or absence of 10 ng/ml recombinant mouse IL-5. Data presented are the means  $\pm$  SEM of three independent observations. Statistical significance was determined using Student-Newman-Keuls comparison testing. Significant differences are indicated ( $p < 0.001$ ).

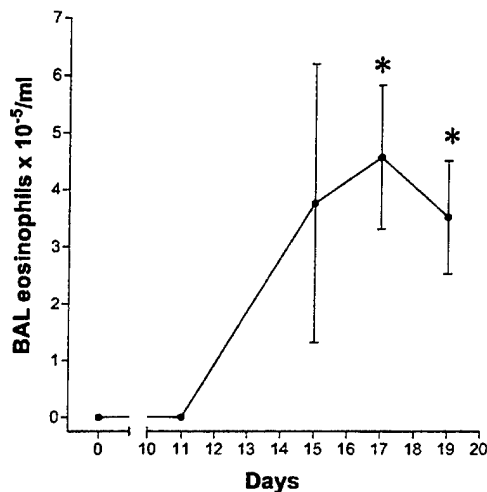
significant elevations in neutrophils (day 11, data not shown), eosinophils (days 15–19, Fig. 5), and macrophages (days 11–17, data not shown). Histopathology of lung tissue samples obtained from saline control mice did not reveal detectable infiltration of inflammatory cells (Fig. 6). However, OVA-exposed mice developed substantial eosinophilic alveolar inflammation (Fig. 6). Histologic evaluation revealed bronchial changes in mice exposed to i.n. OVA, including secretory cell hypertrophy and hyperplasia (Fig. 7). Eosinophilic infiltration was consistently observed in perivascular spaces of the lung (Fig. 7).

#### *Effect of allergen sensitization on bone marrow eosinophil populations in T cell-deficient mice*

To determine the requirement for T lymphocytes in observed alterations of bone marrow CFU-eo following allergen sensitization, athymic nude mice were exposed to OVA using exactly the same protocol described for wild-type BALB/c mice (Fig. 8). Unlike observations in euthymic BALB/c mice, BALB/c nude mice had



**FIGURE 4.** Kinetics of peripheral blood eosinophils during allergen sensitization. Peripheral blood was obtained from each animal and a total white blood cell count was established using a Coulter counter. Peripheral smears were stained with May-Grünwald-Giemsa and a minimum of 200 white blood cells was counted. Data presented are the means  $\pm$  SEM of three independent observations. Statistical significance was determined using Student-Newman-Keuls comparison testing. Statistically significant changes occurred at day 15 ( $p < 0.001$ ), day 17 ( $p < 0.01$ ), and day 19 ( $p < 0.001$ ).



**FIGURE 5.** Kinetics of BAL eosinophils during allergen sensitization. The trachea of mice was cannulated and BAL performed as described. White blood cells were counted under visual microscopy using a hemocytometer. Lavage fluid smears were stained with May-Grünwald-Giemsa and a minimum of 200 white blood cells were counted. Data presented are the means  $\pm$  SEM of three independent observations and are representative of three identical experiments. Statistical significance was determined using ANOVA and Kruskal-Wallis testing. Statistically significant differences were found on days 17 and 19 ( $p < 0.05$ ).

significantly elevated numbers of bone marrow CFU-eo immediately following i.n. allergen exposure (Fig. 8) and CFU-eo remained elevated in athymic mice on day 16.

To determine the duration of this elevation of CFU-eo following allergen exposure in athymic mice, a second series of experiments enumerated CFU-eo through day 19 of the experimental protocol.

The elevation of CFU-eo in the bone marrow of nude mice resolved to baseline numbers 8 days following i.n. exposure to allergen or experimental day 19 (data not presented).

Athymic BALB/c<sup>nu/nu</sup> mice and euthymic BALB/c<sup>+/+</sup> mice did not differ in numbers of bone marrow eosinophils before treatment. However, at 3 days following i.n. exposure to allergen (experimental day 13), the number of bone marrow eosinophils rose dramatically in BALB/c<sup>+/+</sup> mice but remained unchanged in BALB/c<sup>nu/nu</sup> mice (Fig. 8). No differences were found in the total number of nucleated cells in bone marrow of euthymic BALB/c or athymic nude BALB/c mice throughout the experiment (data not shown).

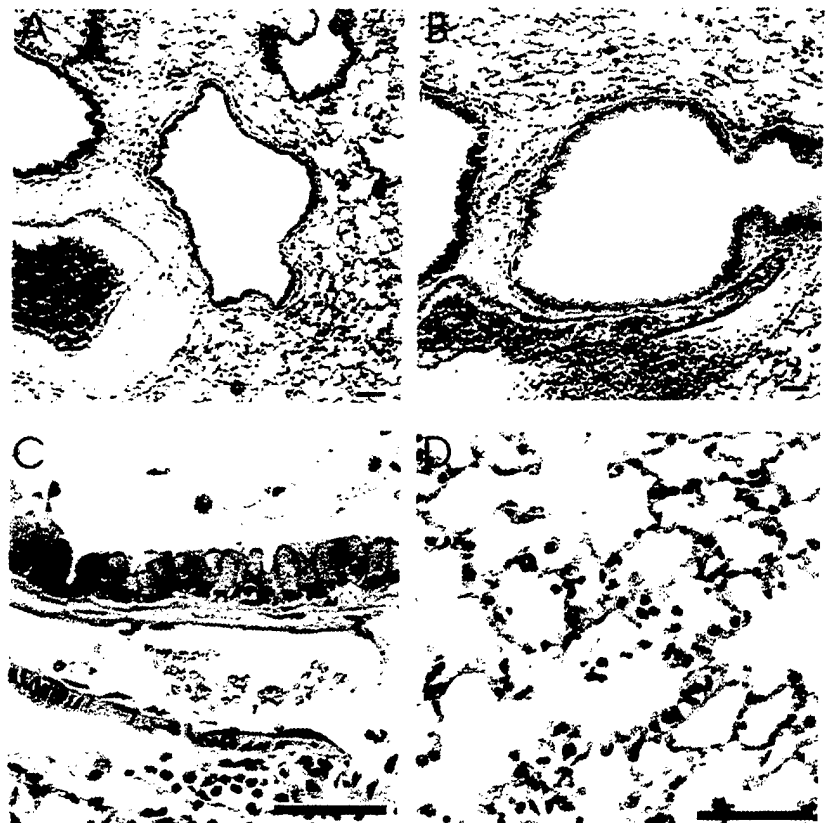
#### *Effect of allergen sensitization on serum levels of anti-OVA IgE*

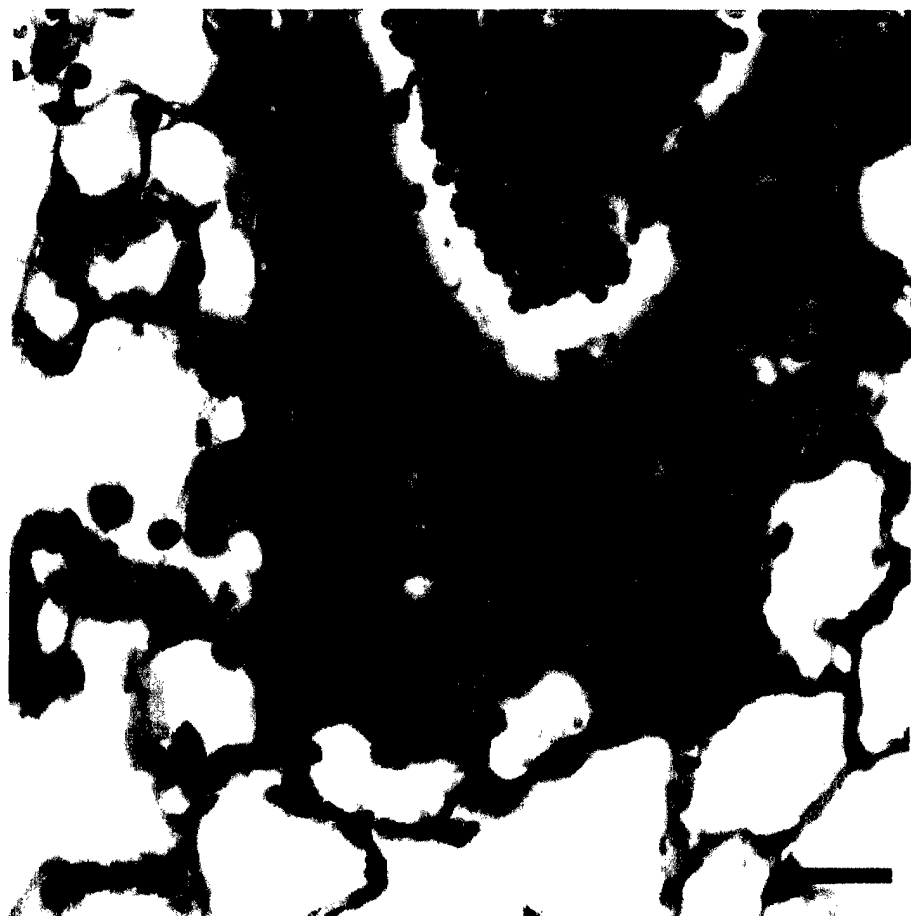
Wild-type BALB/c mice developed anti-OVA IgE Abs over the course of allergen sensitization. On day 11, 30% of BALB/c mice had detectable elevations of IgE and by day 13, 83% had developed OVA-specific IgE Ab. By day 15, all BALB/c mice tested had detectable circulating levels of anti-OVA IgE Abs. None of the athymic BALB/c nude mice in this study developed detectable anti-OVA IgE Abs (observations made on days 13 and 16).

#### *Role of IL-5 in CFU-eo expansion in BALB/c<sup>nu/nu</sup> mice*

It was important to determine whether CFU-eo expansion in nude mice was due to IL-5 produced by cells other than T cells. We determined the number of IL-5-secreting cells in the bone marrow of euthymic and athymic BALB/c mice using ELISPOT analysis to capture IL-5 secreted from individual cells. As shown in Fig. 9, IL-5-producing cells were detected in the bone marrow of both mouse strains; however, there were significantly more IL-5-secreting cells in the bone marrow of wild-type mice as compared with age- and sex-matched nude mice. To determine differences in total IL-5-secreting cells between these mice, cells were also stimulated

**FIGURE 6.** Comparison of pulmonary inflammation between control and OVA-sensitized mice. *A*, Bronchiole and perivascular space in a representative control mouse. *B*, Bronchiole and perivascular space in an OVA-sensitized mouse. *C*, Higher magnification of secretory cell hypertrophy and hyperplasia in an OVA-sensitized mouse. *D*, Mild macrophage and eosinophilic alveolitis in an OVA-sensitized mouse. Bar, 50  $\mu$ m.





**FIGURE 7.** Photomicrograph of pulmonary eosinophil infiltration. Infiltration of the perivascular space by a population of inflammatory cells principally comprised of eosinophils. Bar, 20  $\mu$ m.

with PMA and ionomycin before evaluation in the ELISPOT assay. There was a statistically significant increase in the number of IL-5-producing cells in both euthymic and athymic mice following stimulation with PMA; however, differences between nude and wild-type BALB/c mice continued to be detectable (data not presented).

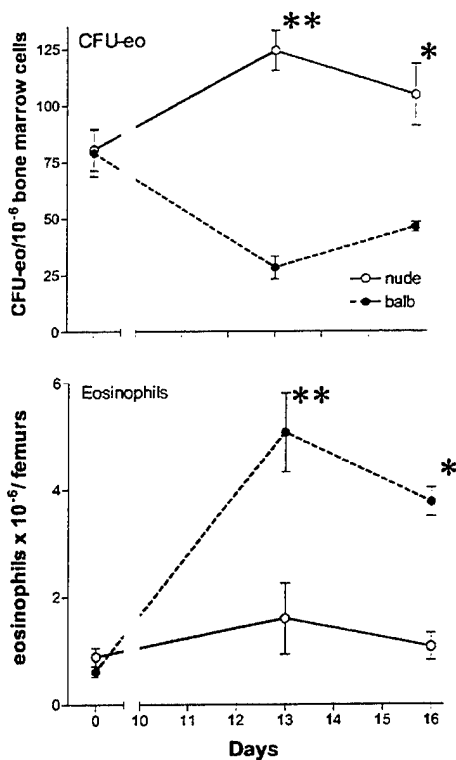
To determine the effect of IL-5 on expansion of bone marrow CFU-eo, nude mice were treated with saturating concentrations of neutralizing Ab to IL-5 *in vivo* (50  $\mu$ g/day *i.p.*) during *i.n.* exposure to allergen and evaluated 4 days later. TRFK-5 anti-IL-5 Ab treatment did not alter expansion of CFU-eo in nude mice exposed to *i.n.* allergen (Fig. 10). However, when the same batch of TRFK-5 Ab was added to *in vitro* bone marrow cultures, it completely neutralized IL-5-mediated formation of CFU-eo colonies (Fig. 11).

## Discussion

Development of asthma in humans or mice is characterized by pulmonary eosinophilia and progressive tissue damage caused by eosinophilic inflammation. Eosinophils are produced in the bone marrow of mammals and recent observations in both mice and humans suggest that pulmonary allergen exposure results in both increased output of eosinophils from hemopoietic tissues and increased migration of these cells to the lung. These observations suggest that alterations of bone marrow function in response to allergen exposure may be a primary factor in understanding progression of asthmatic disease. The purpose of the present study was to use an established animal model of asthma to evaluate alterations of bone marrow function that accompany allergen sensitization and to determine hemopoietic regulatory mechanisms

that are affected by pulmonary allergen exposure. These studies revealed that the population dynamics of eosinophil progenitor cells in the bone marrow is altered following the initial *i.n.* exposure to allergen. These changes in eosinophilopoiesis preceded development of allergen-specific IgE and were, in part, independent of T cell function. Taken together with previous data from this and other laboratories, these studies suggest a working model of bone marrow response to allergen in which bone marrow stromal cells and T lymphocytes act in concert to initiate eosinophilia of asthma (Fig. 12).

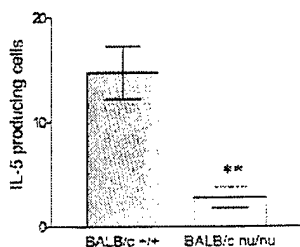
Other laboratories have described altered bone marrow function in response to pulmonary allergen challenge in mice, dogs, and humans (6, 12–16). These studies have largely focused on the response of bone marrow in later stages of asthmatic eosinophilia following development of allergen-specific IgE and the potential role of T cells in alterations of bone marrow function (9, 12–15). We have now evaluated eosinophil development in bone marrow early in the development of asthma and describe a characteristic temporal alteration of eosinophilopoiesis that resulted in increased eosinophil output at this early stage of disease. Following the first *i.n.* installation of allergen in BALB/c mice, the bone marrow eosinophil compartment expanded rapidly and was significantly different from control animals within 72 h following allergen exposure. This increase in bone marrow eosinophils was transient and returned to normal values 7 days following pulsed allergen exposure (Fig. 2). This pattern of bone marrow eosinophilia following allergen sensitization is in general agreement with previous studies (10); however, unlike previous reports, we did not detect differences in overall bone marrow cellularity following allergen exposure at any of the time points tested. Bone marrow eosinophilia



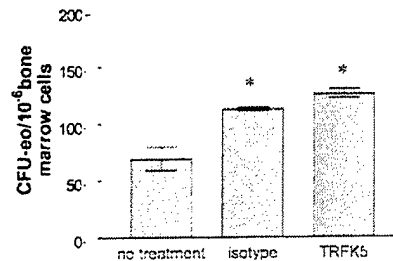
**FIGURE 8.** Comparison of kinetics of bone marrow CFU-eo and eosinophils in euthymic and athymic BALB/c mice during allergen sensitization. Euthymic BALB/c<sup>+/+</sup> or athymic BALB/c<sup>nu/nu</sup> (nude) mice were treated with OVA and alum as described. Bone marrow CFU-eo and eosinophil numbers were enumerated as described. Data presented are the means  $\pm$  SEM of three independent observations and are representative of three identical experiments. Statistical significance was determined using Student-Newman-Keuls comparison testing. Bone marrow CFU-eo were significantly different between euthymic and athymic nude BALB/c mice on day 13 ( $p < 0.001$ ) and day 16 ( $p < 0.01$ ). Bone marrow eosinophil numbers were significantly different between euthymic and athymic nude BALB/c mice on day 13 ( $p < 0.001$ ) and day 16 ( $p < 0.01$ ).

was followed by peripheral blood (Fig. 4) and pulmonary (Fig. 5) eosinophilia on day 5 following exposure, suggesting a plausible temporal sequence of events leading to accumulation of eosinophils in the lung during onset of disease.

Of particular interest to our laboratory, eosinophil progenitor cells (CFU-eo) declined during the first 3 days following the initial i.n. installation of allergen, then rebounded to significantly greater than normal numbers for a period of 48 h before returning to con-



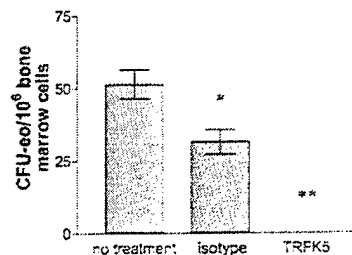
**FIGURE 9.** IL-5-producing cells in bone marrow of nude mice. IL-5-producing cells per 10<sup>6</sup> total cells were enumerated in the bone marrow of BALB/c<sup>+/+</sup> and BALB/c<sup>nu/nu</sup> mice using TRFK-5 anti-IL-5 Ab in ELISPOT analysis to identify positive cells. Data presented are the mean  $\pm$  SE of three independent observations and are representative of four identical independent experiments. Statistical differences were evaluated using ANOVA and Student-Newman-Keuls comparison testing of means ( $p < 0.001$ ).



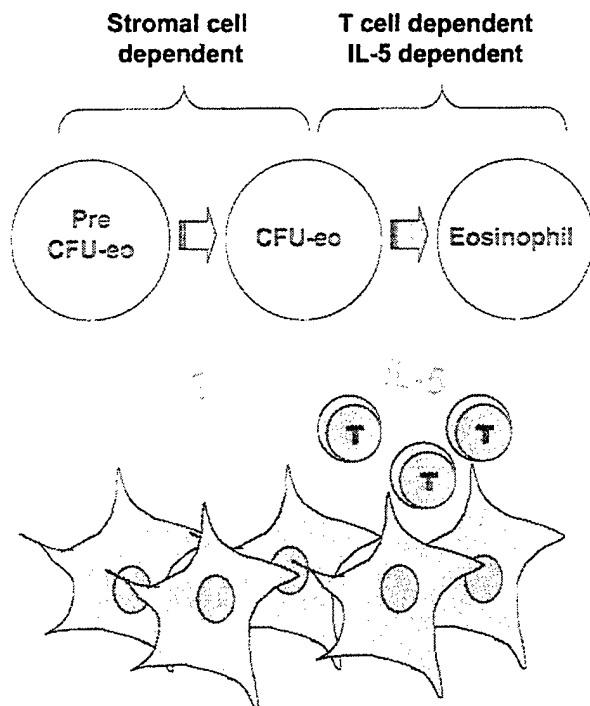
**FIGURE 10.** Effect of TRFK-5 Ab on expansion of CFU-eo in nude mice. BALB/c<sup>nu/nu</sup> mice treated in vivo with 50  $\mu$ g TRFK-5 anti-IL-5 Ab or an isotype-matched control Ig i.p. 1 day before i.n. exposure to allergen and daily for 3 days following i.n. exposure to allergen. Mice were evaluated on day 14 and CFU-eo were enumerated. Statistical differences were evaluated using ANOVA and Tukey-Kramer comparison testing of means. CFU-eo were significantly elevated in mice administered TRFK-5 Ab ( $p < 0.05$ ) or an isotype-matched control Ig ( $p < 0.05$ ). Data presented are the means  $\pm$  SE of three replicate observations.

trol levels (Fig. 1). This pattern of perturbation of hemopoietic progenitor cells has been previously documented in erythropoietic recovery following exposure to hyperbaric conditions (17) and in myeloid progenitors following chemotherapy (18). In both cases, increased demand for end cells resulted in initial depletion, followed by rebound of specific hemopoietic progenitor cells and data presented here suggests that perturbations of eosinophilopoiesis in the bone marrow follows a similar sequence of events. It is interesting to note that, although nasal exposure to allergen in these studies was characterized by pulmonary neutrophilia, no differences in granulocyte-macrophage progenitors (CFU-GM, data not shown) were detected during these early phases of pulmonary allergen exposure in any of the experiments reported here. These observations suggest that increased pulmonary immigration of neutrophils may be more due to redistribution of cells from circulation than altered bone marrow production.

In previous studies, we identified a role for bone marrow stromal cells in regulation of eosinophil production in the bone marrow. However, the relative contribution of stromal cells and T lymphocytes to bone marrow response to allergen has remained unclear. In the studies reported here, we determined the role of T cells in altered bone marrow function by repeating these experiments in T cell-deficient nude mice. In the absence of T lymphocytes, bone marrow eosinophilia did not result from allergen exposure. However, eosinophil progenitor cells (CFU-eo) were dramatically elevated, and this elevation occurred earlier in nude mice than in fully



**FIGURE 11.** Effect of TRFK-5 Ab on development of CFU-eo in vitro. Bone marrow from BALB/c<sup>nu/nu</sup> mice was cultured at limiting dilution in methylcellulose in the presence of IL-5 and CFU-eo were enumerated in the presence or absence of 50  $\mu$ g/ml TRFK-5 anti-IL-5 Ab. Statistical differences were evaluated using ANOVA and Tukey-Kramer comparison testing of means. Data presented are the means  $\pm$  SE of three replicate observations.



**FIGURE 12.** Regulation of eosinophilopoiesis in the bone marrow. Our data suggest a working model of eosinophil production in the bone marrow in which progenitor cell (CFU-eo) renewal and eosinophil production are regulated by separable mechanisms. Proliferation of committed eosinophil progenitor cells (CFU-eo) and maturation of those cells to mature phenotype are regulated by IL-5. IL-5 is produced by both Th2 cells (T) and stromal cells in the bone marrow microenvironment and steady-state eosinophil production is maintained in the absence of T lymphocytes. However, T cells are required for eosinophilia in response to i.n. allergen exposure. Renewal of CFU-eo from hemopoietic stem cells is regulated by bone marrow stromal cells by, as yet, poorly understood mechanisms. Increased CFU-eo production following allergen exposure is T cell and IL-5 independent.

immunocompetent mice. These studies suggest two distinct regulatory processes: with expansion of eosinophil progenitor cells following pulmonary allergen exposure being T cell independent and subsequent proliferation and maturation of expanded progenitor cells to form functional eosinophils being T lymphocyte dependent.

The role of T lymphocytes in development of asthma is well documented. CD4<sup>+</sup> T cells contribute to inflammatory changes observed in lung following pulmonary allergen challenge (19) and both Th1 and Th2 cells participate in this process (20). CD8<sup>+</sup> T cells have also been implicated in the development of airway hyperresponsiveness associated with asthma (21, 22) and this role for T cells appears to be independent of production of specific IgE-mediated Ab responses (23–25).

IL-5 is a critical cytokine in development of eosinophils (26) and previous studies have concluded that IL-5 detected in the marrow is produced by T lymphocytes (11, 14). Previous studies from our laboratory documented that bone marrow stromal cells also produce IL-5 and potentially regulate steady-state production of eosinophils in the absence of asthmatic disease (27). This hypothesis is supported by the presence of normal numbers of eosinophils in athymic nude mice in the present study. However, although we have shown that IL-5 mRNA and protein in stromal cells is elevated by exposure to IL-1, an inflammatory mediator associated with asthma, eosinophil production was not altered by pulmonary allergen exposure in T cell-deficient mice. These data suggest that

regulation of both the progenitor cell compartment and phenotypic maturation to functional end cells may be multifactorial and more complex than previously described.

The finding that CFU-eo were increased following allergen challenge in the absence of T cells suggests that the primary role of stromal cells may be in regulation of the compartment size of eosinophil progenitor cells (CFU-eo) in response to pulmonary inflammation. Although stromal cells produce IL-5 (27) in the bone marrow microenvironment, the observation that observed expansion of CFU-eo in nude mice following sensitization to OVA was not affected by daily administration of a neutralizing Ab to IL-5 suggests that IL-5 is likely not to be the cytokine primarily responsible for CFU-eo expansion in response to allergen exposure. We also noted that nude mice had little alteration of eosinophil output, even though cells other than T cells produce IL-5 in these mice (Fig. 9). This failure of IL-5 production to stimulate increased numbers of eosinophils may be due to the relative levels of IL-5 released by T lymphocytes and stromal cells, the sequestration of cytokine on stromal cell surfaces, or the presence of inhibitors of cell differentiation known to be produced by bone marrow stromal cells. Surprisingly, we noted in ELISPOT assays that the amount of IL-5 captured on plates did not differ between normal and nude mice and, therefore, there is no evidence for a difference in the amount of IL-5 produced per cell in these mice (data not presented).

These experiments confirm that allergen-specific IgE is not required for the bone marrow CFU-eo response to allergen during sensitization. Changes in bone marrow CFU-eo populations occurred in the absence of detectable OVA-specific IgE Ab in athymic mice. In addition, maximal alteration of CFU-eo was documented in immunocompetent BALB/c mice on day 13, a time at which only 30% of animals had detectable OVA-specific IgE Ab.

The finding that eosinophil progenitor proliferation and subsequent eosinophil differentiation are regulated by separable mechanisms is consistent with data for other developing hemopoietic cell lineages. We previously reported that early development of B lymphoid progenitors was T cell independent and required the presence of bone marrow stromal cells (28–31). However, differentiation of pre-B cells in the bone marrow to form functional B lymphocytes depended on the presence of IL-4, a T cell-derived cytokine (28, 32). The present study presents a similar working hypothesis for the production of eosinophils in the bone marrow and suggests that stromal cell regulation of eosinophil progenitor cell expansion is independent of both T cells and IL-5 production. Defining the identity of cytokines and cellular interactions which regulate early events in this lineage will be essential to understanding the role of bone marrow in the allergic response to allergen.

The role of tissue inflammation in regulation of hemopoiesis is not well understood. We previously demonstrated that elevated levels of IL-1 or IL-4 altered bone marrow stromal cell function and production of B lymphocytes in that tissue (32). Our recent work has extended that observation to eosinophilopoiesis. Bone marrow stromal cells produce the primary eosinophilopoietic cytokine, IL-5, and IL-5 abundance in stromal cells increased when stromal cells were exposed *in vitro* to rIL-1 (27). This increase in IL-5 production by stromal cells was shown to be correlated with increased eosinophil production *in vitro*. However, the present study strongly suggests that stromal cells regulate eosinophil progenitor cell expansion in the bone marrow by an IL-5-independent mechanism and that this regulatory function is also elevated in response to airway inflammation. Taken together, these studies

support the hypothesis that systemic release of inflammatory mediators may serve as a primary regulatory stimulus for altered hemopoietic response to immune insult, including alterations of bone marrow function known to result from pulmonary allergen exposure.

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